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**Maliga**

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(54) **SELECTABLE MARKER GENE AND METHODS OF USE THEREOF IN TRANSPLASTOMIC PLANTS**

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CPC ..... **C12N 15/8214** (2013.01); **C12N 15/8212** (2013.01)

(58) **Field of Classification Search**  
CPC ..... **C12N 15/8214**  
See application file for complete search history.

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(57) **ABSTRACT**

Compositions and method for the generation of transplastomic plants are provided.

**11 Claims, 12 Drawing Sheets**

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Figure 1

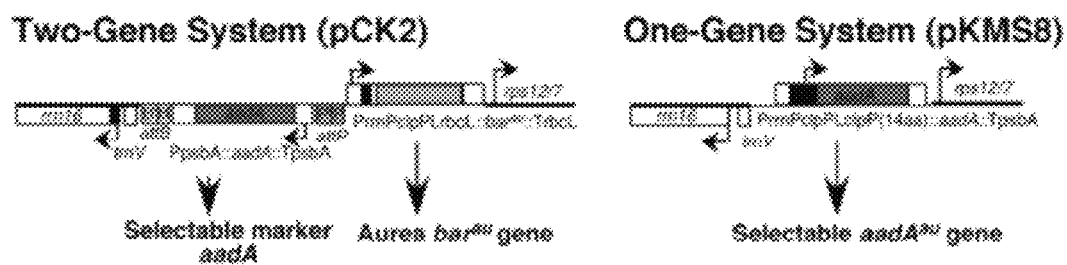


Figure 2

(A)

Plasmid	Chimeric genes	Aurea phenotype	No. genes per pDNA
pSS42		++++	2
pSS52		+	2
pKMS10		++	2
pKMS8		++++	2
pKMS12		+++	1

(B)

PrmPcIpP::Lrbcl (pCK2, pSS42 and pSS52)

```

1 gagctcGCTC CCCCGCCGTC GTTCAATGAG AATGGATAAG AGGCTCGTGG
51 GATTGACGTG AGGGGGCAGG GATGGCTATA TTTCTGGCAG TTACGTTTCC
101 ACCTCAAAGT GAAATATAGT ATagttgtag ggagggatccATGG

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PrmPcIpP+DB (pHK32, pKMS8 and pKMS12)

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1 gagctcGCTC CCCCGCCGTC GTTCAATGAG AATGGATAAG AGGCTCGTGG
51 GATTGACGTG AGGGGGCAGG GATGGCTATA TTTCTGGCAG TTACGTTTCC
101 ACCTCAAAGT GAAATATAGT ATTTAGTTCT TTCTTTCATT TAATGCCAT
151 TGGTOTTCCA AAAGTCCCTT TCCGAAGTCC TCGAGAGCAA gctagc

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PrmPcIpP (pHK33 and pKMS10)

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1 gagctcGCTC CCCCGCCGTC GTTCAATGAG AATGGATAAG AGGCTCGTGG
51 GATTGACGTG AGGGGGCAGG GATGGCTATA TTTCTGGCAG TTACGTTTCC
101 ACCTCAAAGT GAAATATAGT ATTTAGTTCT TTCTTTCATT TAATGCCAT
151 tagc

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Figure 3

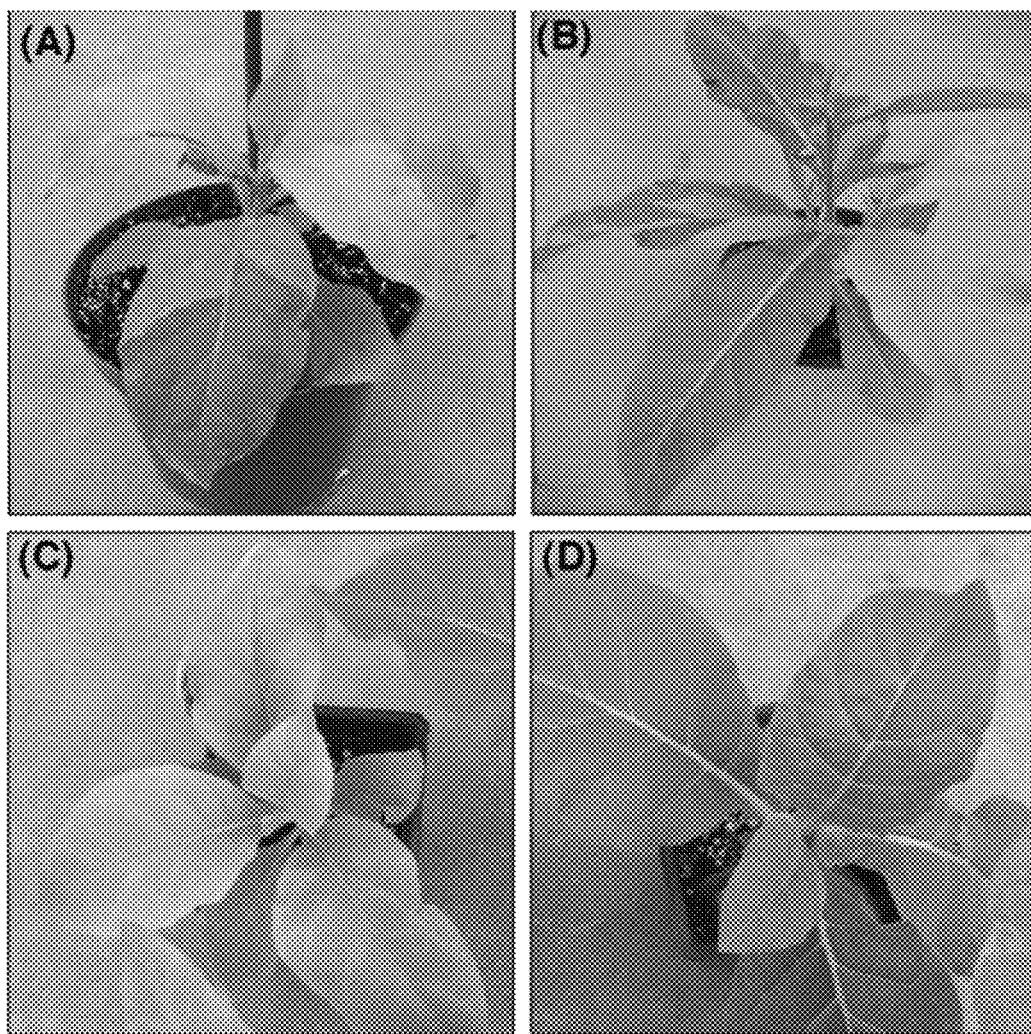
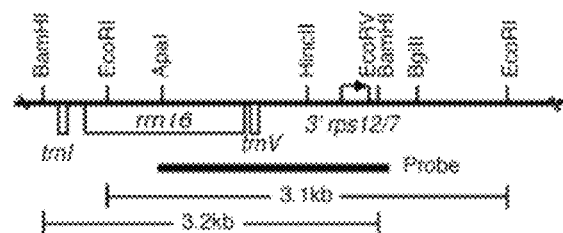
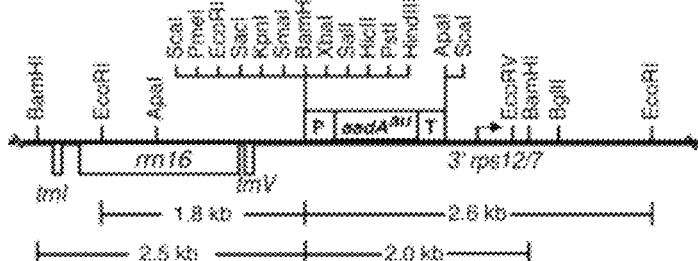


Figure 4

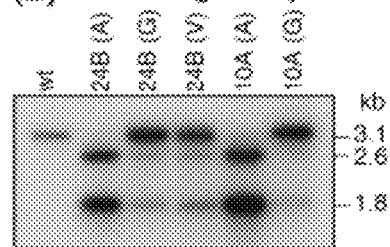
## (A) wt-ptDNA



## Nt-pKMS8



## (B) EcoRI digested pt-DNA (1X regenerated)



## BamHI digested pt-DNA (2X regenerated)

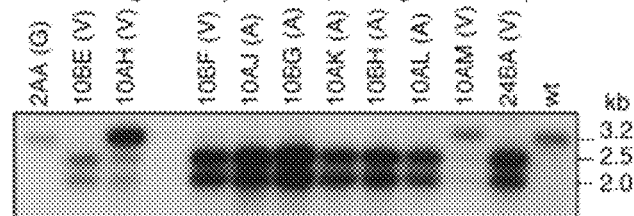


Figure 5

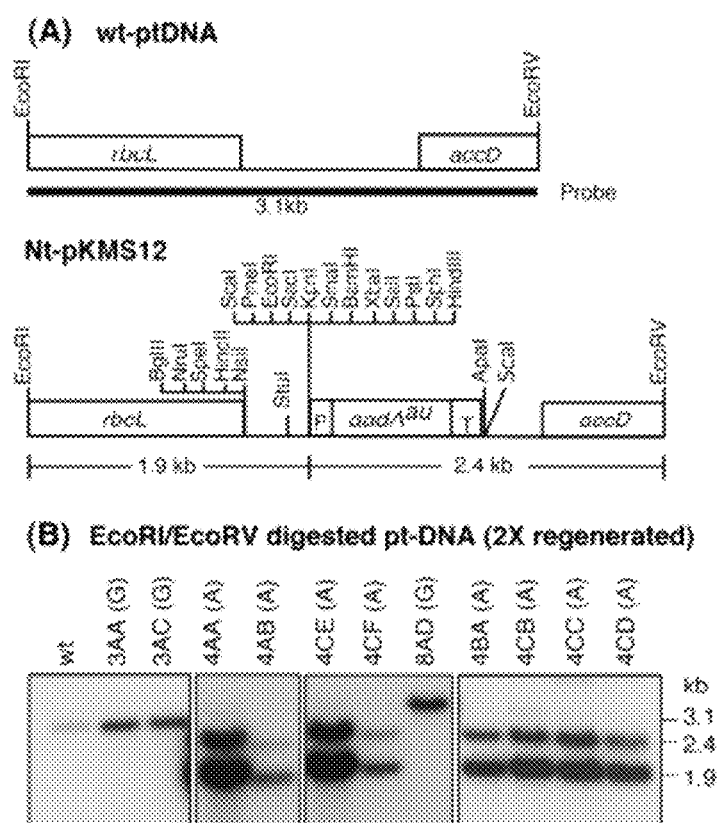


Figure 6

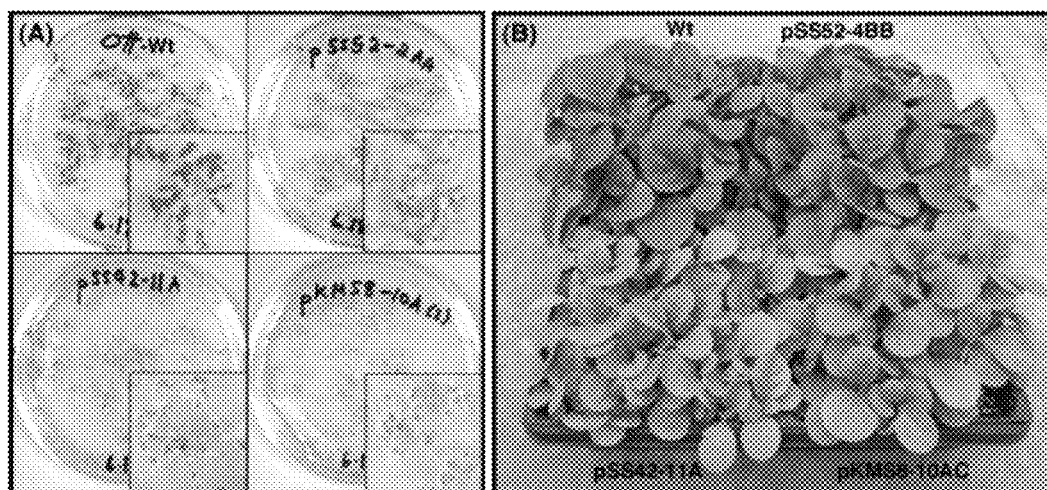




Figure 7

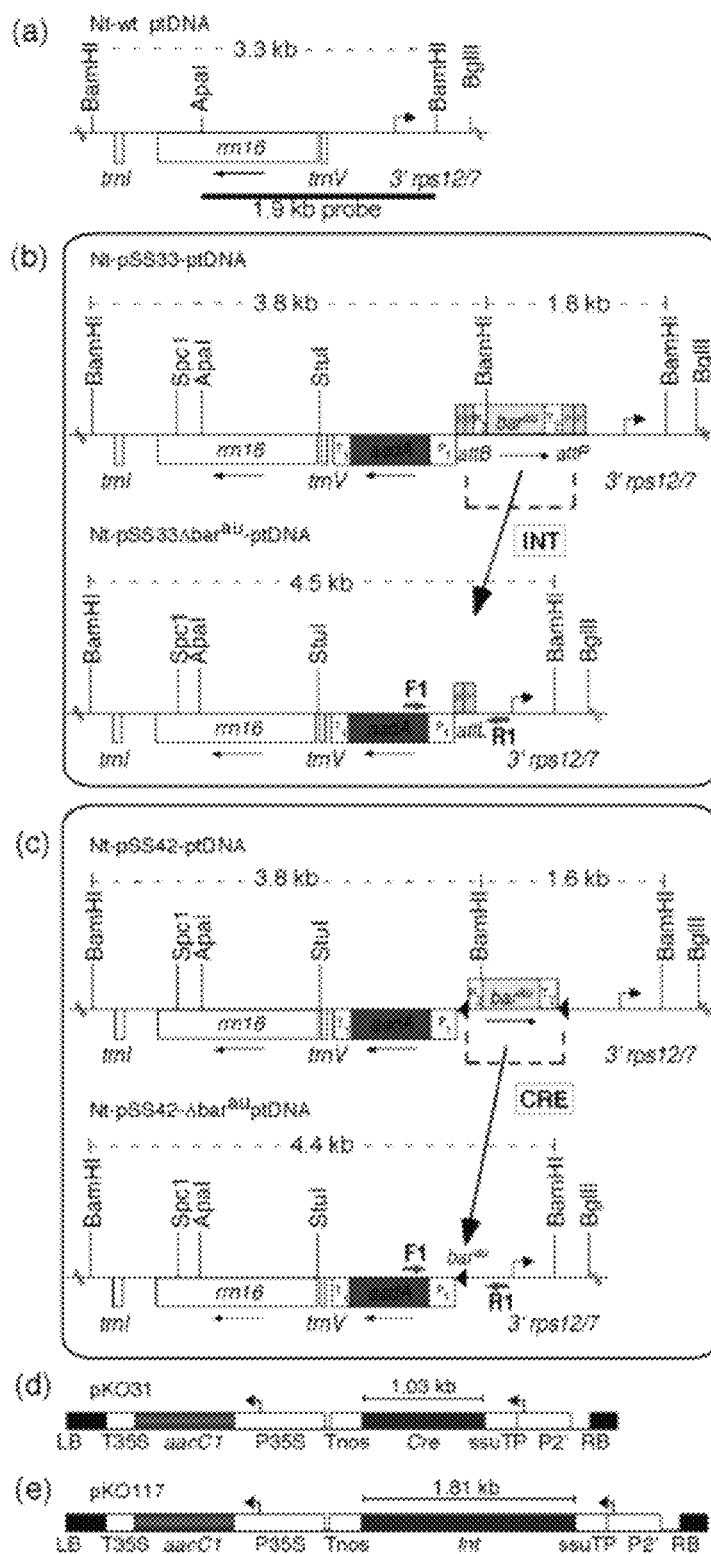
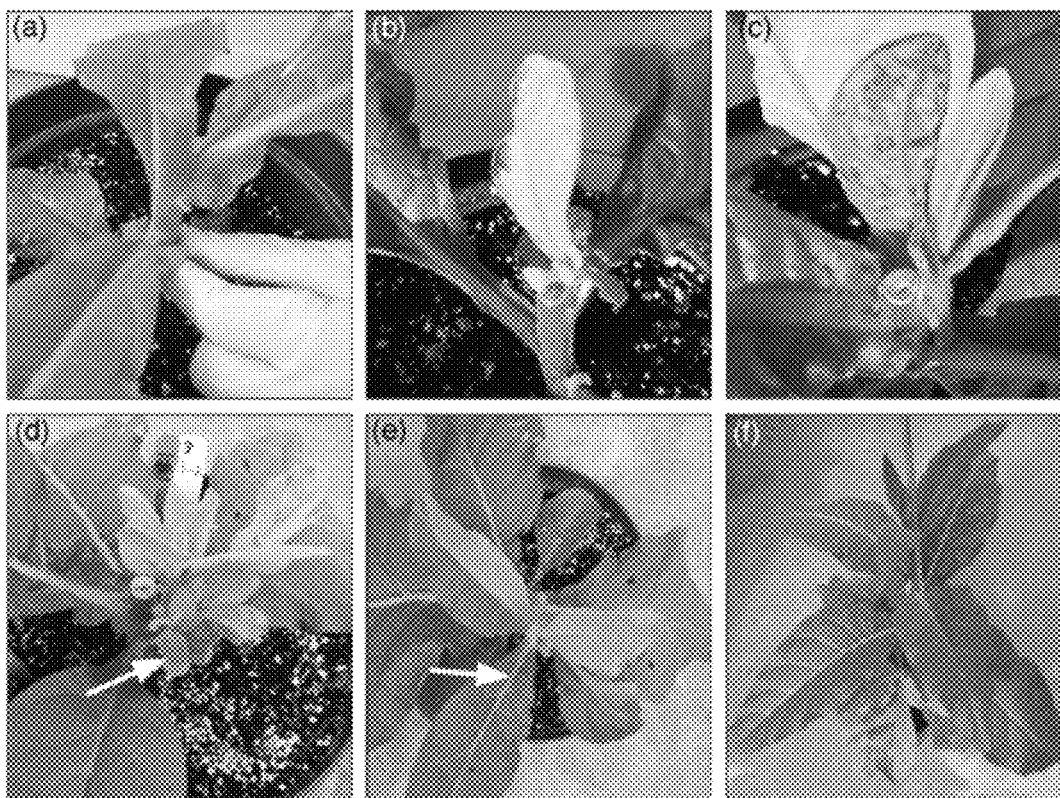


Figure 8



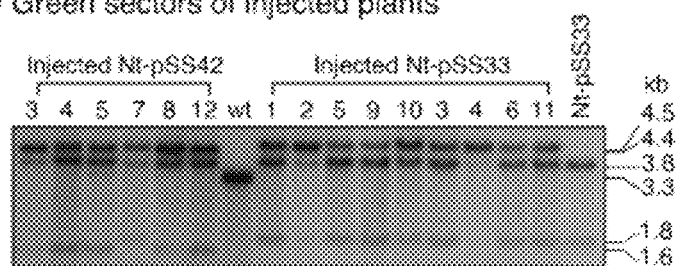
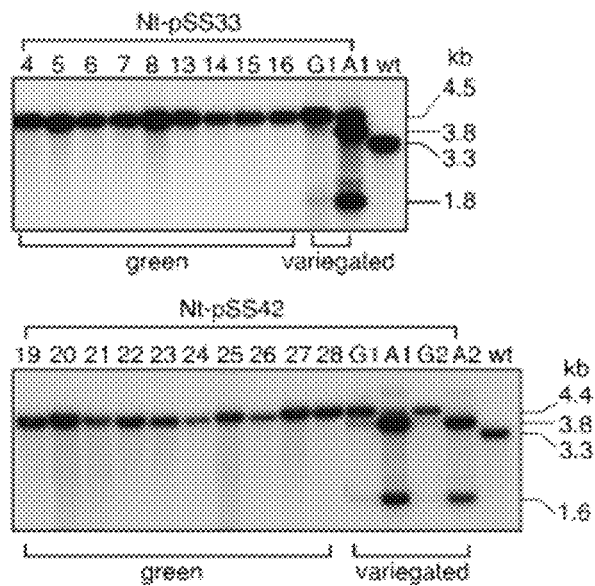
**Figure 9****(a) Green sectors of injected plants****(b) Seed progeny of injected plants**

Figure 10

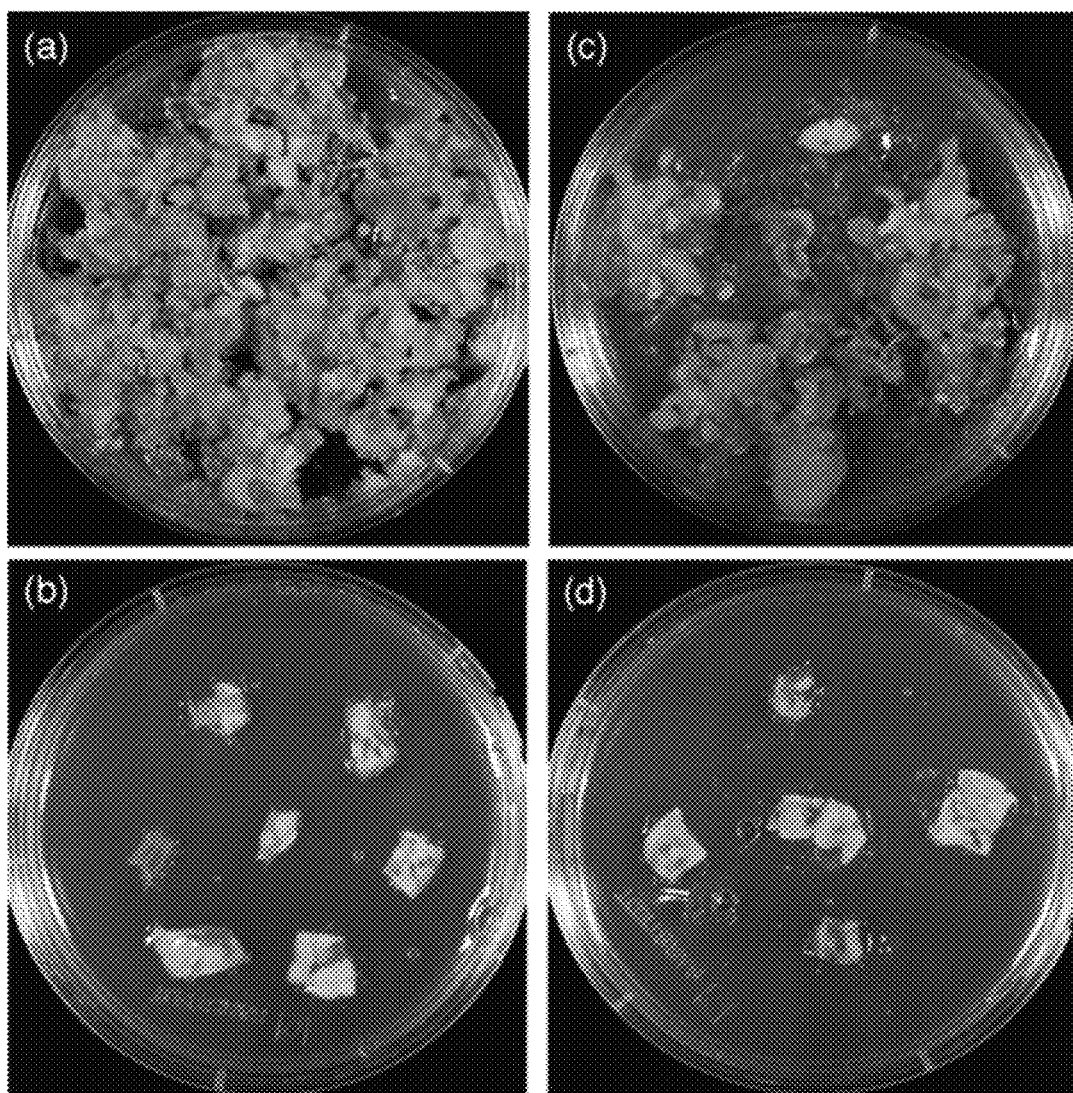
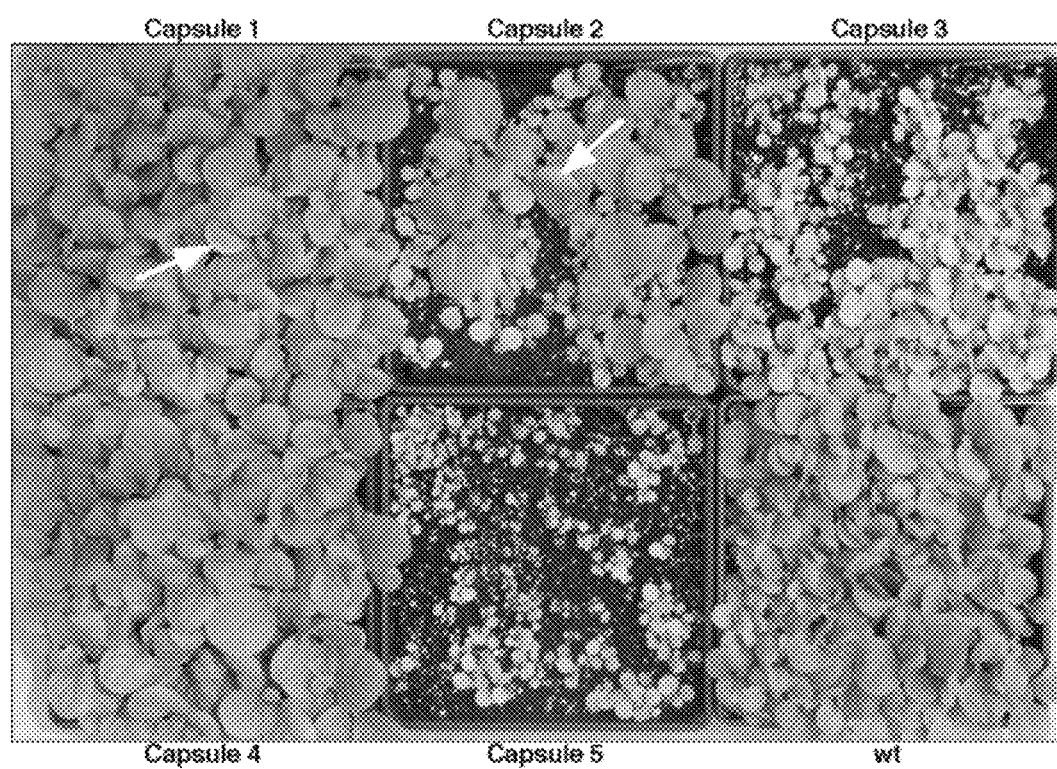


Figure 11



Visual pt-Vector

T-ptDNA exc

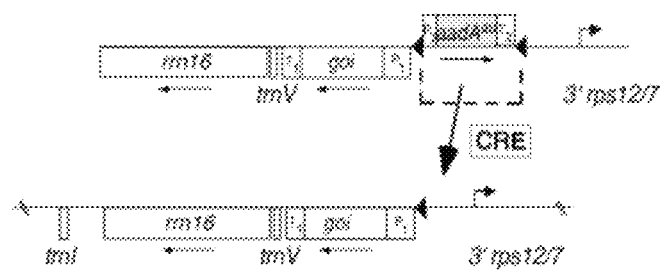


Figure 12

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## SELECTABLE MARKER GENE AND METHODS OF USE THEREOF IN TRANSPLASTOMIC PLANTS

This Application claims priority to U.S. Provisional Application No. 61/422,952 filed Dec. 14, 2010, the entire disclosure being incorporated herein by reference as though set forth in full.

Pursuant to 35 U.S.C. §202(c) it is acknowledged that the U.S. Government has rights in the invention described, which was made in part with funds from the USDA National Institute of Food and Agriculture Biotechnology Risk Assessment Research Grant Program, Grant Numbers 2005-33120-16524 and 2008-03012.

### FIELD OF THE INVENTION

This invention relates to the fields of molecular biology and transplastomic plants. More specifically the invention provides a selectable marker encoding nucleic acid and methods of use thereof for the production of transgenic plants having desirable characteristics.

### BACKGROUND OF THE INVENTION

Several publications and patent documents are cited throughout the specification in order to describe the state of the art to which this invention pertains. Each of these citations is incorporated herein by reference as though set forth in full.

Angiosperm plastids carry a relatively small genome (ptDNA), which is 120 kb to 160 kb in size and encodes ~130 genes (Raubeson and Jansen 2005). These genes include some, but not all genes required for plastid transcription and translation. In addition, plastid genes encode photosystem, ATPase and NDH subunits, a gene involved in protein degradation and another in lipid biosynthesis (Wakasugi, et al. 2001). Transformation of the plastid genome is employed to probe plastid gene function in knockout plants and to beneficially alter processes localized to plastids, for example photosynthesis (Sharwood, et al. 2008, Whitney and Andrews 2001, Whitney and Andrews 2003), lipid biosynthesis (Madoka, et al. 2002) and the biosynthesis of vitamins (Apel and Bock 2009). Transformation is also used to incorporate novel genes in the ptDNA for the production of industrial enzymes and pharmaceutical proteins. For reviews see (Bock 2007, Daniell, et al. 2005, Maliga 2004).

Plastid transformation is based on homologous recombination between the ptDNA and ptDNA fragments in the vectors flanking a marker gene. Because plant cells contain hundreds to thousands of ptDNA copies, selective amplification of the transformed ptDNA copy is important for the recovery of transplastomic clones. According to the commonly used protocol, the tobacco leaves are bombarded with DNA-coated gold particles, then the leaves are cut into small pieces and transferred to a shoot regeneration medium containing spectinomycin. The selective medium suppresses greening and shoot regeneration of wild type cells and the transplastomic clones are identified as green shoots. The shoots regenerating from the bombarded leaves are chimeric. Genetically stable plants with a uniform population of transformed ptDNA (homoplastomic plants) are obtained by regenerating new shoots from the chimeric leaves. Typically two cycles of such purifying regeneration are required to obtain homoplastomic plants. Marker genes available in plastids for selective enrichment confer resistance to spectinomycin and streptomycin (aadA) (Svab and Maliga

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1993), kanamycin (neo or aph(3')IIa) (Caner, et al. 1993, Huang, et al. 2002) (Lutz, et al. 2004), chloramphenicol (Li, et al. 2011) or the amino acid analogues 4-methylindole (4MI) and 7-methyl-DL-tryptophan (7MT) (ASA2) (Barone, et al. 2009). Because the plants that are expressing marker genes have no visual phenotype, homoplastomic state can be verified only by DNA gel blot analyses.

It is clear that selectable marker genes conferring a visibly detectable phenotype are highly desirable. It is an object of the invention to provide such marker genes.

### SUMMARY OF THE INVENTION

In accordance with the present invention an isolated nucleic acid encoding a visibly detectable selectable marker for identification of transformed plastids wherein said nucleic acid encodes aadA<sup>+</sup> is provided. In another embodiment, the nucleic acid is contained within a vector suitable to transform higher plants. In yet another aspect, the vector may comprise a heterologous nucleic acid encoding a protein of interest. Plant cells transformed with the nucleic acid or vectors described herein are also encompassed by the present invention as are plants comprising the same.

In yet another embodiment, methods for obtaining transplastomic plants are disclosed. An exemplary method entails introducing the nucleic acid encoding the visibly detectable marker gene into a plant cell and selecting those transformed cells which exhibit the golden leaf phenotype and spectinomycin resistance, regenerating shoots from said transformed cells, and rooting said shoots in soil under conditions suitable to generate said transplastomic plant. In one approach, the nucleic acid is introduced using a suitable plastid transformation vector which can optionally encode one or more heterologous proteins of interest. Plants obtained by the foregoing methods are also within the scope of the present invention.

In another aspect of the invention, a method for in planta selectable marker gene excision is disclosed. An exemplary method comprises providing a plant comprising plastids transformed with a nucleic acid encoding a selectable marker gene, the marker gene comprising a nucleic acid sequence which upon effective excision from plastids, confers a phenotype on a plant cell or plant sector that is identifiable via visual inspection, the nucleic acid being flanked by excision sites. A nucleic acid encoding a recombinase which acts on said excision sites is delivered directly to cells positioned in the plant body of a) to form a shoot apex under conditions suitable for recombinase expression, thereby effecting recombinase mediated excision of the selectable marker gene. Those sectors which lack the selectable marker gene and as a result exhibit a dark green color in cells which contribute to the germline of progeny plants and at least one bud and/or branch associated therewith are visually selected; and seed is then collected. Also within the scope of the invention are plants obtained from the method described above and seed and progeny obtained from said plant. In a preferred embodiment, the selectable marker gene is present in a vector. In a particularly preferred embodiment, the vector is pKMS8 or pKMS12 which further comprise excision sites which flank said selectable marker gene.

In another embodiment of the invention, a method for in planta plastid transformation is disclosed. An exemplary method entails providing a nucleic acid construct encoding i) a selectable marker gene operably linked to ii) a sequence which upon expression confers a phenotype on a plant cell or plant sector that is identifiable via visual inspection

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operably linked to iii) a nucleic acid encoding a heterologous nucleic acid encoding a protein of interest, sequence; wherein i, ii, and iii are flanked by plastid DNA sequences which enable homologous recombination into the plastids of the plant to be transformed. In the method, the construct is delivered to cells positioned in the plant body to form a shoot apex and shoot formation is induced in said plant. Sectors which exhibit golden leaf phenotype and least one bud and/or branch associated therewith are then selected and seed collected from resulting branches. Seed so produces also provide an embodiment of the invention.

#### BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 The two-gene and new one-gene aurea marker systems. In the two-gene pCK2 vector *bar<sup>au</sup>* confers the aurea phenotype and *aadA* is the selective marker (Kittiwongwattana, et al. 2007). In the new one-gene aurea vector pKCMS8 *aadA<sup>au</sup>* performs both roles: confers the aurea phenotype and is the selective marker. Flanking ptDNA encoding the *rrn16* and *trnV* genes (on the left) and *rps12/7* (on the right) target the insertion of transgenes by homologous recombination.

FIG. 2 Chimeric *aadA* genes to induce aurea phenotype in plants. a *Aurea<sup>au</sup>* genes have unique 5' regulatory sequences (FIG. 2b) and share the *aadA* coding region (fused with c-myc tag; filled box) along with TpsbA (3'-UTR or transcription terminator). On top is the reference aurea *bar<sup>au</sup>* gene encoded in plasmid pSS42 (Tungsuchat-Huang, et al. 2010). The black box schematically depicts *clpP1* sequence. The intensity of aurea phenotypes (four to one plus marks for the most to least intense) and the number of *aadA<sup>au</sup>* gene copies per ptDNA are also listed. b DNA sequence of 5'regulatory regions of aurea *aadA<sup>au</sup>* genes (SEQ ID NOs: 1-3 from top to bottom). Nucleotides comprising the *rrn16* PEP promoter (*Prn*) (Suzuki, et al. 2003) are in capital letter and underlined; the *clpP1*-53 NEP promoter (*PclpP*), the *ClpP* leader and *ClpP* protease subunit coding sequences are in capital letters (not underlined); the minimal *clpP1*-53 promoter that was defined in plasmid pPS45 (Sriraman, et al. 1998) is boxed. Within *Prn*, the plastid rRNA upstream activator (RUA, GTGGGA; italics) and *rrn16* promoter-35 (TTGACG; bold) and -10 (TATATT; bold) sequences are marked (Suzuki, et al. 2003). Aurea genes in plasmids pCK2 and pSS52 have the *rbcL* leader (nucleotides in lower case); in plasmids pKMS12 the *clpP1* leader; (nucleotides in capital letters); and in pKMS8 and pKMS12 the *clpP1* leader and 14 amino acids of the coding region N-terminus (*PclpP*+DB; nucleotides in capital letters). Plasmids, which carry transgenes with the same 5'-regulatory region are listed including: pCK2 (Kittiwongwattana, et al. 2007); pSS42 (Tungsuchat-Huang, et al. 2010); pHK32 (unpublished); and pHK33 (Kuroda and Maliga 2002).

FIG. 3 The aurea phenotypes of greenhouse plants from the first purifying regeneration. a,b Variegated Nt-pKMS8 plants and Nt-pKMS12 plants with c aurea and d green phenotype.

FIG. 4 DNA gel blot analyses of Nt-pKMS8 leaves provide confirmation that the aurea sectors contain transformed plastids. a Maps of the transformed ptDNA in Nt-pKMS8 plants and the cognate region in wild type ptDNA. Map position of the plastid *rrn16*, *trnV* and 3'-*rps12/7* leader and the probe are shown. b DNA gel blot analyses of variegated leaf sectors. Leaf phenotypes are given in parenthesis above the lanes. Abbreviations: A, aurea; G, green; V, variegated.

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FIG. 5 DNA gel blot analyses of Nt-pKMS 12 leaves confirm that the aurea sectors contain transformed plastids. a Maps of the transformed ptDNA in Nt-pKMS12 plants and the cognate region in wild type ptDNA. b DNA gel blot analyses of leaves. The blots were probed with the 3.1 kb EcoRI-EcoRV fragment (FIG. 7a). Leaf phenotypes are given in parenthesis above the lanes. Abbreviations: A, aurea; G, green.

FIG. 6 The aurea phenotype of transplastomic seedlings. Shown are a two-week old seedlings germinated in sterile culture on MS medium (100 mm×20 mm Petri dish) and b 27-day old seedlings grown in greenhouse (20"×20" tray). Note that Nt-pSS52 seedlings have an aurea phenotype in sterile culture but are similar to wild type (Wt) in the greenhouse. Note also that the Nt-pSS42 and Nt-pKMS8 seedlings have comparable phenotypes in culture and in the greenhouse.

FIG. 7 Visual system to detect recombinase-mediated plastid marker excision. (a) Engineered region in the wild type ptDNA. Shown are the *rrn16*, *trnV* and 3'-*rps12* plastid genes and relevant restriction endonuclease cleavage sites. (b) Nt-pSS33 transplastome with *aadA* spectinomycin resistance gene and *bar<sup>au</sup>* flanked with the attB-attP target sites and the product of *bar<sup>au</sup>* excision by *Int*. Cassettes driving the expression of transgenes are: P1 and T1, the *psbA* plastid gene promoter and terminator; and P2 is the *Prn**PclpP* promoter and T2 the *rbcL* terminator. F1 and R1 mark the position of primers to sequence the recombinant target site. (c) Nt-pSS42 transplastome with the *aadA* gene and *bar<sup>au</sup>* flanked with loxP target sites and the product of *bar<sup>au</sup>* excision by *Cre*. For further explanation see caption to FIG. 1b. (d,e) T-DNA region in plasmids pKO31 and pKO117 with a plastid-targeted *Cre* and *Int*, respectively. The recombinases are expressed in the P2 promoter-Tnos terminator cassettes. Shown are also the left and right T-DNA borders and the *aacC1* plant-selectable gentamycin resistance gene.

FIG. 8 Green sectors form in aurea leaves after *Agrobacterium* injection. (a) *Agrobacterium* injection of the aurea Nt-pSS42 tobacco stem, at the bud site. Note that only the rapidly developing, young leaves have the aurea color and the older leaves shown here turn green. (b-d) Excision of the *bar<sup>au</sup>* gene by *Cre* in the Nt-pSS42 plants yields a few (b) or many (c,d) dark green sectors (arrow). (e) Half of the shoot apex of Layer 2 is dark green in Nt-pSS33 plant injected with *Agrobacterium* carrying plasmid pKO117. (d) Forcing shoot development from the axillary bud in FIG. 2e by decapitating shoot above the dark green leaf (arrow in FIG. 2e) yields uniform dark green shoot.

FIG. 9. DNA analyses confirm marker excision in green leaves. Total cellular DNA was digested with the *Bam*HI restriction endonuclease and probed with the *Apal*-*Bam*HI targeting region fragment. Predicted *Bam*HI fragment sizes are shown in FIG. 1a-c. (a) DNA gel blot analyses of green sectors in the Agroinjected plants. Note that all samples are heteroplastomic due to chimeric leaf structure or mixed plastids. (b) DNA gel blot analyses of Agroinjected Nt-pSS33 and Nt-pSS42 seed progeny. Lanes contained DNA from individual seedlings of Nt-pSS33(I3-2, lanes 4, 13-16, G1/A1; 13-5, lanes 5-8) and Nt-pSS42(I11-1, lanes 25-28; I11-11, lanes 19,20; I3-6, lanes 21-24, G1/A1, G2/A2).

FIG. 10 Testing gentamycin resistance in green leaf sectors. (a) Formation of green callion the selective medium indicates gentamycin resistance and integration of recombinase gene. (b) Sensitivity to gentamycin suggests *bar<sup>au</sup>* excision by transiently expressed recombinase. (c) Segregation for gentamycin resistance in different explants of the



same sector suggests excision by both mechanisms. (d) Wild-type gentamycin sensitive cultures.

FIG. 11 Seed transmission of the green (*bar<sup>au</sup>*-free) plastids to the Nt-pSS33-IBA-I3-2 seed progeny. Seed from individual capsules (1-5) were sown in trays. Green and variegated seedlings grew faster than their aurea sibs. Seedling DNA analyses (FIG. 3b) confirmed *bar<sup>au</sup>* excision. White arrow in Capsule 2 seedlings points to variegated leaf. For reference, wild-type (wt) seedlings are also shown.

FIG. 12. In planta plastid transformation vector with the *bar<sup>au</sup>* visual marker gene. On top is shown the vector with the *bar<sup>au</sup>* gene flanked by loxP sites (triangles) and a gene of interest (*goi*) and the left targeting region encoding the *rrn16* and *trnV* plastid genes and the right targeting region encoding the 3'-*rps12/7* operon promoter. Cassettes driving the expression of transgenes are: P1 and T1, the *psbA* plastid gene promoter and terminator; P2 is the *PrmPclpP* promoter and T2 the *rbcL* terminator. Below is shown the transplastome, after excision of the *bar<sup>au</sup>* by Cre.

#### DETAILED DESCRIPTION OF THE INVENTION

Identification of a genetically stable *Nicotiana tabacum* (tobacco) plant with a uniform population of transformed plastid genomes (ptDNA) takes two cycles of plant regeneration from chimeric leaves and analysis of multiple shoots by Southern probing in each cycle. Visual detection of transgenic sectors facilitates identification of transformed shoots in the greenhouse, complementing repeated cycles of blind purification in culture. In addition, it provides a tool to monitor the maintenance of transplastomic state. Our current visual marker system requires two genes: the aurea *bar<sup>au</sup>* gene that confers a golden leaf phenotype and a spectinomycin resistance (*aadA*) gene that is necessary for the introduction of the *bar<sup>au</sup>* gene in the plastid genome. We developed a novel *aadA* gene that fulfills both functions: it is a conventional selectable *aadA* gene in culture, and allows detection of transplastomic sectors in the greenhouse by leaf color.

Common causes of pigment deficiency in leaves are mutations in photosynthetic genes, which affect chlorophyll accumulation. We use a different approach to achieve pigment deficiency: post-transcriptional interference with the expression of the *clpP1* plastid gene by aurea *aadA<sup>au</sup>* transgene. This interference produces plants with reduced growth and a distinct color, but maintains a wild-type gene set and the capacity for photosynthesis. Importantly, when the aurea gene is removed, green pigmentation and normal growth rate are restored. Because the aurea plants are viable, the new *aadA<sup>au</sup>* genes are useful to query rare events in large populations and for in planta manipulation of the plastid genome.

The following definitions are provided to aid in understanding the subject matter regarded as the invention.

"Heteroplastomic" refers to the presence of a mixed population of different plastid genomes within a single plastid or in a population of plastids contained in plant cells or tissues.

"Homoplastomic" refers to a pure population of plastid genomes, either within a plastid or within a population contained in plant cells and tissues. Homoplastomic plastids, cells or tissues are genetically stable because they contain only one type of plastid genome. Hence, they remain homoplastomic even after the selection pressure has been removed, and selfed progeny are also homoplastomic. For purposes of the present invention, heteroplastomic popula-

tions of genomes that are functionally homoplastomic (i.e., contain only minor populations of wild-type DNA or transformed genomes with sequence variations) may be referred to herein as "functionally homoplastomic" or "substantially homoplastomic." These types of cells or tissues can be readily purified to a homoplastomic state by continued selection.

"Plastome" refers to the genome of a plastid.

"Transplastome" refers to a transformed plastid genome.

Transformation of plastids refers to the stable integration of transforming DNA into the plastid genome that is transmitted to the seed progeny of plants containing the transformed plastids.

A "selectable marker gene" refers to a gene that upon expression confers a phenotype by which successfully transformed plastids or cells or tissues carrying the transformed plastid can be identified. Selectable marker genes as used herein can confer resistance to a selection agent in tissue culture and/or confer a phenotype which is identifiable upon visual inspection. Thus, in one embodiment the selectable marker gene can act as both the selection agent and the agent which enables visual identification of cells comprising transformed plastids. In an alternative embodiment, the selectable marker encoding nucleic acid comprises two sequences, one encoding a molecule that renders cells resistant to a selection agent in tissue culture and another that enables visual identification of cells comprising transformed plastids.

Transforming DNA refers to homologous DNA, or heterologous DNA flanked by homologous DNA, which when introduced into plastids becomes part of the plastid genome by homologous recombination.

"Agroinfiltration" refers to *Agrobacterium* mediated T-DNA transfer. Specifically, this process involves vacuum treatment of leaf segments in an *Agrobacterium* suspension and a subsequent release of vacuum, which facilitates entry of bacterium cells into the inter-cellular space.

"T-DNA" refers to the transferred-region of the Ti (tumor-inducing) plasmid of *Agrobacterium tumefaciens*. Ti plasmids are natural gene transfer systems for the introduction of heterologous nucleic acids into the nucleus of higher plants:

A "plant sector" refers to a region or a full leaf of a plant that is visually identifiable due to expression of a selectable marker gene or the excision of a selectable marker gene in accordance with the present invention.

"Operably linked" refers to two different regions or two separate genes spliced together in a construct such that both regions will function to promote gene expression and/or protein translation.

"Nucleic acid" or a "nucleic acid molecule" as used herein refers to any DNA or RNA molecule, either single or double stranded and, if single stranded, the molecule of its complementary sequence in either linear or circular form. In discussing nucleic acid molecules, a sequence or structure of a particular nucleic acid molecule may be described herein according to the normal convention of providing the sequence in the 5' to 3' direction. With reference to nucleic acids of the invention, the term "isolated nucleic acid" is sometimes used. This term, when applied to DNA, refers to a DNA molecule that is separated from sequences with which it is immediately contiguous in the naturally occurring genome of the organism in which it originated. For example, an "isolated nucleic acid" may comprise a DNA molecule inserted into a vector, such as a plasmid or virus vector, or integrated into the genomic DNA of a prokaryotic or eukaryotic cell or host organism.

When applied to RNA, the term "isolated nucleic acid" refers primarily to an RNA molecule encoded by an isolated

DNA molecule as defined above. Alternatively, the term may refer to an RNA molecule that has been sufficiently separated from other nucleic acids with which it would be associated in its natural state (i.e., in cells or tissues). An isolated nucleic acid (either DNA or RNA) may further represent a molecule produced directly by biological or synthetic means and separated from other components present during its production.

The terms “percent similarity”, “percent identity” and “percent homology” when referring to a particular sequence are used as set forth in the University of Wisconsin GCG software program.

The term “functional” as used herein implies that the nucleic or amino acid sequence is functional for the recited assay or purpose.

The phrase “consisting essentially of” when referring to a particular nucleotide or amino acid means a sequence having the properties of a given SEQ ID No.: For example, when used in reference to an amino acid sequence, the phrase includes the sequence per se and molecular modifications that would not affect the basic and novel characteristics of the sequence.

A “replicon” is any genetic element, for example, a plasmid, cosmid, bacmid, phage or virus, that is capable of replication largely under its own control. A replicon may be either RNA or DNA and may be single or double stranded.

A “vector” is a replicon, such as a plasmid, cosmid, bacmid, phage or virus, to which another genetic sequence or element (either DNA or RNA) may be attached so as to bring about the replication of the attached sequence or element.

An “expression operon” refers to a nucleic acid segment that may possess transcriptional and translational control sequences, such as promoters, enhancers, translational start signals (e.g., ATG or AUG codons), polyadenylation signals, terminators, and the like, and which facilitate the expression of a polypeptide coding sequence in a host cell or organism.

The term “oligonucleotide,” as used herein refers to primers and probes of the present invention, and is defined as a nucleic acid molecule comprised of two or more ribo- or deoxyribonucleotides, preferably more than three. The exact size of the oligonucleotide will depend on various factors and on the particular application and use of the oligonucleotide.

The term “probe” as used herein refers to an oligonucleotide, polynucleotide or nucleic acid, either RNA or DNA, whether occurring naturally as in a purified restriction enzyme digest or produced synthetically, which is capable of annealing with or specifically hybridizing to a nucleic acid with sequences complementary to the probe. A probe may be either single-stranded or double-stranded. The exact length of the probe will depend upon many factors, including temperature, source of probe and use of the method.

The term “primer” as used herein refers to an oligonucleotide, either RNA or DNA, either single-stranded or double-stranded, either derived from a biological system, generated by restriction enzyme digestion, or produced synthetically which, when placed in the proper environment, is able to functionally act as an initiator of template-dependent nucleic acid synthesis.

All amino-acid residue sequences represented herein conform to the conventional left-to-right amino-terminus to carboxy-terminus orientation.

The term “tag,” “tag sequence” or “protein tag” refers to a chemical moiety, either a nucleotide, oligonucleotide, polynucleotide or an amino acid, peptide or protein or other chemical, that when added to another sequence, provides

additional utility or confers useful properties, particularly in the detection or isolation, to that sequence.

The terms “transform”, “transfect”, “transduce”, shall refer to any method or means by which a nucleic acid is introduced into a cell or host organism and may be used interchangeably to convey the same meaning. Such methods include, but are not limited to, transfection, electroporation, microinjection, PEG-fusion, biolistic bombardment and the like.

A “clone” or “clonal cell population” is a population of cells derived from a single cell or common ancestor by mitosis.

A “cell line” is a clone of a primary cell or cell population that is capable of stable growth in vitro for many generations.

The following materials and methods are provided to facilitate the practice of the present invention.

#### Plastid Transformation Vectors

We describe four new plasmids in Example I, pSS52, pKMS8, pKCMS10 and pKCMS12 carrying promoter variants of aurea *aadA<sup>au</sup>* genes. DNA sequence of the promoters is shown in FIG. 2b. Plasmids pSS52, pKMS8, pKCMS10 are pPRV1-II vector derivatives targeting insertions of *aadA<sup>au</sup>* between the *trnV* and 3'-*rps12* plastid genes localized in the repeat region of the ptDNA. Plasmid pKMS12 is a pSS22 vector (FJ416604) derivative targeting insertion of the *aadA<sup>au</sup>* in the large single copy region between the *rbcl* and *accD* genes. Plastid genomes transformed with plasmids pKMS 12 carry one copy of *aadA<sup>au</sup>*, while plastids transformed with plasmids pSS52, pKMS8 and pKMS10 carry two copies of *aadA<sup>au</sup>*. The promoters drive the expression of a c-myc tagged *aadA* coding region (SEQ ID NO: 7) and *psbA* 3'-UTR. DNA sequence of the *aadA<sup>au</sup>* gene is deposited in GenBank under accession number HQ023426 as part of plasmid pKMS8 DNA sequence (SEQ ID NO: 6).

#### Plastid Transformation

The *Nicotiana tabacum* cv. Petit Havana plastid genome was transformed using the biolistic protocol as previously described (Lutz and Maliga 2007, Lutz, et al. 2006, Svab and Maliga 1993). Briefly, 0.6  $\mu$ m gold particles were coated with plasmid DNA and nine plates containing tobacco leaves were bombarded with each plasmid using the Du Pont PDA1000He biolistic gun. Transplastomic clones were selected on RMOP medium containing 500 mg l<sup>-1</sup> spectinomycin dihydrochloride. Shoots developing on the selective medium were dissected and rooted on MS medium containing salts and 3% sucrose (Murashige and Skoog 1962).

#### Testing the Aurea Phenotype

Plants were transferred to soil (ProMix General Purpose Growing Medium Code 0432, Premier Horticulture Inc., Grower Services, Quakertown, Pa. 18951) in the greenhouse and fertilized weekly with a liquid solution of Blu-Grow 10-10-10 General Purpose Liquid Fertilizer (Plant Food Company, Cranbury, N.J. 08512) supplemented with monthly applications of Osmocote Classic 14-14-14 Controlled Release Fertilizer (Scotts-Sierra Horticultural Products Co., Marysville, Ohio 43401). Seeds were collected 5-6 months after the plants had been transferred to soil. Seedling phenotype in the greenhouse was scored three weeks after sowing in soil. Seedling phenotype in culture was evaluated two weeks after germinating seed on the surface of MS medium (salts plus 3% sucrose) (Murashige and Skoog 1962) in 100x20 mm Petri dishes. The seeds were vapor sterilized for 3 hours in open Eppendorf tubes in a desiccation chamber above a mix of 100 ml commercial bleach and 3 ml of concentrated hydrochloric acid.

## Plastid DNA Analysis

Plastid DNA analysis has been described in previous protocols (Lutz and Maliga 2007, Lutz, et al. 2006). Briefly, total leaf cellular DNA was isolated from leaves by the CTAB protocol (Murray and Thompson 1980) and digested with appropriate restriction endonucleases. The DNA fragments were separated by electrophoresis in 0.8% agarose gels and then transferred to Hybond-N membranes (GE healthcare, UK). Hybridization was carried out in Church buffer (Church and Gilbert 1984). Double stranded DNA probes were the 2.0-kb *Apal*-*Bam*HI fragment containing the *rrn16* gene and the 3.1-kb *Swa*I-*Swa*I fragment from plasmid pSS16 (Sinagawa-Garcia, et al. 2009). The probes were labeled using the Ready-To-Go DNA Labeled Beads (dCTP) (Amersham Pharmacia Biotech UK Ltd., Amersham 9NA).

The following example is provided to facilitate the practice of the present invention. It is not intended to limit the invention in any way.

## Example 1

Visual Spectinomycin Resistance (*aadA<sup>au</sup>*) Gene for Facile Identification of Transplastomic Sectors in Tobacco LeavesEngineering the *rrn16-clpP1* Dual Promoter to Induce an Aurea Phenotype

The first promoter that yielded a pigment deficient aurea phenotype was serendipitously discovered in Nt-pHK33 transplastomic plants when testing translation efficiency from the plastid rRNA operon promoter (*Prn*P1) fused with the 5' untranslated region (5'-UTR) of the plastid *clpP1* gene (Kuroda and Maliga 2002). Because the *PclpP* 1-53 promoter in plasmid pHK33 initiates transcription upstream the promoter (Sriraman, et al. 1998), the kanamycin resistance (*neo*) gene in Nt-pHK33 plastids is expressed from two promoters: the *Prn*P1 promoter initiating transcription downstream (Suzuki, et al. 2003) and the *PclpP*1-53 promoter initiating transcription upstream the promoter consensus. The two promoters overlap at a G nucleotide that is the transcription initiation site in both promoters (FIG. 2b). The mild aurea phenotype of Nt-pHK33 plants was attributed to reduced availability of the *ClpP1* protease subunit (Kuroda and Maliga 2002). To minimize the aurea phenotype, the size of the *clpP1* fragment in plasmid pCK2 (Kittiwongwattana, et al. 2007) was trimmed to the minimal *PclpP*1-53 promoter, as it is present in plasmid pPS45 (Sriraman, et al. 1998). The aurea phenotype, instead of being weaker, became more prominent in the Nt-pCK2 plants. The difference between the chimeric genes in plasmids pHK33 and pCK2 was not only in the promoter, but also in the coding regions: in plasmid pHK33 the chimeric promoter drives a *neo* gene, in plasmid pCK2 a *bar* gene. We decided to engineer *aadA* to endow it with the ability to confer visual selection. We first expressed an *aadA* gene in the cassette that drives the expression of *bar* in plasmid pCK2. We have found that the Nt-pSS52 plants had only a mild aurea phenotype as compared to the Nt-pSS42 plants (see below), which carry the aurea *bar<sup>au</sup>* gene first described in the Nt-pCK2 plants (Tungsuchat-Huang, et al. 2010). We assumed that the reduced aurea phenotype this time was due to expressing *aadA* instead of the *bar* coding region. Therefore, we created three additional *aadA* constructs, one of which (pKMS10) has the *clpP1* 5'-UTR and two plasmids (pKMS8 and pKMS12) which have the *clpP1* 5'-UTR and

14 amino acids of the *ClpP1* N-terminus that comprises the Downstream Box (DB) sequence (Kuroda and Maliga 2001a, Kuroda and Maliga 2001b, Sprengart, et al. 1996). Plasmids pKMS8 and pKMS12 target insertions in different regions in the ptDNA yielding two and one *aadA<sup>au</sup>* copies per ptDNA, respectively. Double *aadA<sup>au</sup>* gene dosage in the Nt-pKMS8 plants was expected to yield a more robust aurea phenotype. The *aadA<sup>au</sup>* transgenes are schematically shown in FIG. 2a; the 5'-regulatory sequences are given in FIG. 2b.

## Selection of Transplastomic Clones in Culture

The objective was to develop tissue culture selectable spectinomycin resistance genes, which enable detection of transplastomic sectors in greenhouse plants by the golden leaf color. Plasmids pSS52, pKMS8, pKMS10 and pKMS12 carry only the *aadA<sup>au</sup>* gene as selective marker. Transplastomic clones were selected in bombarded leaf cultures as green shoots on the background of bleached wild type cells. The shoots obtained after bombardment with the plasmids were green and indistinguishable in appearance from shoots obtained by other *aadA* genes. The frequency of transplastomic clones was about the same as with other *aadA* genes, up to ~3 clones per bombarded sample. Thus, each of these plasmids passed the first test, providing a suitable marker for the selective recovery of transplastomic clones. We named the plants by the initial of the plant species (Nt), the plasmid name (pKMS8) and a serial number for the independently transformed clone (10), for example Nt-pKMS8-10. One and two letters were added to distinguish plants obtained from the first and second purifying regeneration, respectively, for example Nt-pKMS8-10A and Nt-pKMS8-10AL. Detection of Transplastomic Sectors by Golden Leaf Color

The second criterion for the visual marker was facile detection of transplastomic sectors in greenhouse plants by the golden leaf color. To judge the value of the *aadA<sup>au</sup>* genes as visual markers, plants were transferred to the greenhouse after the first and second purifying regeneration of the putative transplastomic clones. The first purifying regeneration involves shoot regeneration from leaves that are taken from shoots developing on bombarded leaf sections. Purifying regeneration is necessary, because ptDNA is initially transformed in only a few plastids and the shoots regenerating on the selective medium in most cases have only small transplastomic sectors. Cross-protection of wild-type cells carrying no transformed ptDNA by transplastomic tissue enables regeneration of wild type shoots from mixed, chimeric leaves. To directly assess the fraction of plants carrying transplastomic sectors, we rooted shoots from the first and second purifying regeneration and transferred the plants to the greenhouse. Among the fourteen plants derived from the first purifying regeneration of pKMS8-transformed shoots ~2/3 (nine) of the plants had variegated leaves with aurea sectors and 1/3 (five) were green; no uniformly aurea plants were obtained (Table 1) (FIG. 3). DNA gel blot analysis confirmed that the green plants carried wild type ptDNA. The green sectors of variegated leaves carried wild type, or mixed wild type and transplastomic ptDNA (T-ptDNA) while the aurea sectors contained T-ptDNA (FIG. 4). Among the 39 plants derived from the second purifying regeneration of pKMS8-transformed shoots ~40% (sixteen) had an aurea phenotype, ~45% (seventeen) had variegated leaves with aurea sectors and ~15% (six) were green (Table 1). DNA gel blot analyses in 12 out of 13 aurea plants confirmed a uniform population of T-ptDNA (FIG. 4b). A similar trend was observed among plants obtained when transformation was carried out with plasmid pKMS12: most plants from the first purifying regeneration were wild type whereas after the second purifying regeneration about

half of the plants were wild type and half were homoplas-  
tomic transgenic (Table 1) (FIG. 5). The Nt-pKMS12 plants  
had a weaker aurea phenotype than the Nt-pKMS8 plants;  
this we attribute to having one copy of the *aadA<sup>au</sup>* in  
Nt-pKMS12 plastids and two copies in pKMS8.

Although Nt-pKMS 10 plants also had an aurea pheno-  
type, this phenotype was weaker than those of Nt-pKMS12  
plants (three and two plus marks on a subjective scale,  
respectively, see FIG. 2a). What complicates judging the  
aurea phenotype is its transient nature: the golden-yellow  
color is well expressed in rapidly growing leaves and  
disappears in older leaves that turn green as growth slows.  
We therefore recommend the Nt-pKMS8 vector because the  
aurea color of Nt-pKMS8 leaves is maintained until the first  
flower buds appear enabling unambiguous classification of  
plastid genotypes by leaf color.

#### The *aadA<sup>au</sup>* Gene in Nt-pKMS8 Plants Confers an Aurea Seedling Phenotype

Delayed growth and vibrant aurea color facilitate distinc-  
tion of aurea seedlings from wild type. We therefore germi-  
nated Nt-pSS52 and Nt-pKMS8 seed at the same time as the  
Nt-pSS42 seeds carrying the reference aurea *bar<sup>au</sup>* gene and  
wild type seed (FIG. 6). We have found that Nt-pKMS8  
seedlings carrying the pKMS8 *aadA<sup>au</sup>* allele and the refer-  
ence Nt-pSS42 seedlings carrying the reference *bar<sup>au</sup>* gene  
are affected to a similar extent with respect to delay in seed  
germination and greening. The two-week old Nt-pSS52  
seedlings had an intermediate phenotype between the wild  
type and Nt-pKMS8 seedlings in culture, and were similar  
to wild type in the greenhouse when older (27 days).

### DISCUSSION

#### Conversion of the *aadA* Spectinomycin Resistance Gene into a Visual Marker

We report here successful conversion of the *aadA* gene  
into a visual marker so that the presence or absence of the  
transgene can be determined by visual inspection of leaves.  
The *aadA<sup>au</sup>* gene is unique because it combines two roles: it  
is a selectable marker in tissue culture and a visual marker  
in plants. Selection in tissue culture is carried out as with a  
conventional *aadA* gene. Using an aurea *aadA<sup>au</sup>* instead of a  
conventional *aadA* does not shorten the time required for  
plastid sorting and the time required to obtain homoplas-  
tomic plants. Having the visual phenotype has the advantage  
that homoplastic plants can be readily distinguished from  
variegated, heteroplastic plants (FIG. 3). Such varie-  
gated, heteroplastic plants may be present even after two  
cycles of plant regeneration (Table 1). These variegated  
plants remain undetected using the conventional *aadA* gene,  
but are readily identified using the *aadA<sup>au</sup>* gene. The only  
disadvantage using the aurea *aadA<sup>au</sup>* gene is that slows down  
development. The aurea marker is the most useful to query  
rare events in large populations, or when excision of the  
marker gene is planned from the final product (see below).

TABLE 1

Progress towards homoplastic state in regenerated plants				
	Phenotype			
	Nt-pKMS8		Nt-pKMS12	
	Purified 1 x	Purified 2 x	Purified 1 x	Purified 2 x
Green	5	6	19	19
Variegated	9	17	4	0
Aurea	0	16	6	12

Conventional visual markers are based on mutations in  
plastid genes causing pigment deficiency. Pigment defi-  
ciency can be readily induced by targeted deletion of plastid  
genes, such as the RNA polymerase subunit-encoding *rpoB*  
gene (Allison, et al. 1996), and the *rbcL* (Kanevski and  
Maliga 1994) and *psbA* (Baena-Gonzales, et al. 2003)  
photosynthetic genes. These pigment mutants are difficult to  
handle because they are unable to grow without supporting  
wild-type tissue. The interference by the *aadA<sup>au</sup>* marker  
produces plants with reduced growth and a distinct color, but  
maintains a wild-type gene set and the capacity for photo-  
synthesis. More importantly, when the aurea marker is  
removed, normal growth rate signaling the fully functional  
status of plastids is restored (see below). The new marker  
gene will take over the role of the aurea *bar<sup>au</sup>* gene, which  
induces a similar visual phenotype, but is not selectable in  
culture.

Genes conferring kanamycin resistance to plastids, such  
as neo and aph(3')IIa, are selectable in tissue culture (Cane,  
et al. 1993, Huang, et al. 2002, Lutz, et al. 2004). A mild  
form of an aurea phenotype was first discovered as a  
phenotype caused by a neo gene variant encoded in plasmid  
pHK33 (Kuroda and Maliga 2002). We did not consider  
engineering neo to enhance its aurea phenotype because  
inefficient translation from the *clpP1* leader yielded only low  
levels (~0.26%) of the encoded NPTII, considered too low  
for kanamycin selection in culture. In contrast, low levels  
(<1%) of AAD, the *aadA* gene product, are sufficient for the  
recovery of transplastomic clones (Sinagawa-Garcia, et al.  
2009).

A salient feature of the aurea phenotype conferred by  
*aadA<sup>au</sup>* is its transient nature: it is readily detectable in young  
seedlings, presumably reflecting the high demand for the  
*ClpP 1* protease subunit (Kuroda and Maliga 2002) that  
subsides as growth slows and, by the time the plants are  
flowering, wild type pigmentation is restored. Consequently,  
to satisfy the demand for rapid growth, regular fertilization  
is critical when scoring seedlings for the aurea phenotype.  
The *aadA<sup>au</sup>* Gene Enables Visual Assessment of the  
Homoplastic State

We took the opportunity of the visual phenotype of our  
new transplastomic clones to assess the progress towards  
achieving the homoplastic state, when each cell carries a  
uniform population of transformed ptDNA. From the data it  
became apparent that: (i) a significant fraction of shoots can  
be wild type even in the second purifying regeneration cycle  
and that (ii) the aurea phenotype is a reliable predictor of  
plastid genotype. Induction of a new shoot apical meristem  
from one cell is the opportunity to create a new shoot apex  
with a uniform ptDNA population.

Therefore, regeneration of a new shoot apex is essential to  
obtain genetically uniform plants because cells with differ-  
ent plastid types may be locked in different developmental  
layers of the shoot apex. Genetically stable, homoplastic  
plants are obtained when the two to three long-term stem  
cells in each of the three layers carry the same plastid types  
(Lutz and Maliga 2008). To allow time for plastid sorting,  
we normally take older leaves for purifying regeneration  
from well-developed shoots. If plastid sorting is advanced  
and the leaves contain homoplastic sectors of transplas-  
tomic and wild type cells, the regenerated plants will be  
either transplastomic or wild type. Indeed, the variegated  
category was absent in the twice-purified Nt-pKMS 12  
plants that were either homoplastic or wild type, as  
expected (Table 1). However, heteroplastic plants are  
obtained in significant numbers in the second purifying

selection if the first purifying selection was initiated too early, as in the Nt-pKMS8 cultures.

#### Applications of Aurea Plastid Genes

Because the plants are viable, the aurea markers are useful to query rare events in large populations. The recovery of rare events depends on, whether or not small green sectors can be identified on the aurea background. The vectors described here do not carry sequences for marker excision, thus are not suitable to test the restoration of green phenotype, due to excision of *aadA<sup>au</sup>*. However, study of plastid segregation reported here with the *aadA<sup>au</sup>* gene yielded results that are similar to those obtained with the *bar<sup>au</sup>* gene. The examples for past application of the *bar<sup>au</sup>* gene include the study of plastid sorting in heteroplasmic cells, where heteroplasmic cells were obtained by excision mediated by a site-specific recombinase. The study led to the conclusion that ptDNA in a regenerating tobacco shoot derive from a small number of copies selected through a stochastic process. Thus, formation of heteroplasmic plants during shoot regeneration is not necessarily the rule because ptDNA in a regenerating shoot derives from a small number of ptDNA copies and even rare plastid types may sort out to yield a homoplasmic plant (Lutz and Maliga 2008). The *bar<sup>au</sup>* gene was also useful to test plastid genome stability when the marker gene was flanked by direct repeats that are target sites for site-specific recombinases. In this study, the *bar<sup>au</sup>* gene was flanked by loxP or attP/attB target sites that are recognized by the Cre and phiC31 site specific recombinases. Loss of the *bar<sup>au</sup>* gene could have been detected by formation of green seedlings among the aurea plantlets. Because in a population of ~40,000 seedlings no green plants were obtained in which loss of the *bar<sup>au</sup>* gene was due to recombination via the recombinase target sites, it was concluded that the repeats required for marker excision are too short to facilitate deletion of the marker genes at a measurable rate (Tungsuchat-Huang, et al. 2010). When the *bar<sup>au</sup>* gene marker is removed, green pigmentation and normal growth rate are restored so that marker-free plants can be identified as sectors or in the seed progeny. Plants with target site flanked *bar<sup>au</sup>* marker genes were useful to explore in planta marker excision by *Agrobacterium* injection so that seed of plastid marker-free plants could be obtained without using tissue culture. See Example II. Expected uses of the aurea *aadA<sup>au</sup>* gene are the same as of the *bar<sup>au</sup>* gene with the additional advantage that the transplastomic clones can be obtained by direct selection for the *aadA<sup>au</sup>* gene.

#### References for Example I

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## Example II

Visual Marker and *Agrobacterium*-Delivered Recombinase Enable the Manipulation of the Plastid Genome in Greenhouse-Grown Tobacco Plants

Successful manipulation of the plastid genome (ptDNA) so far has been carried out in tissue culture cells, a limitation that prevents plastid transformation being applied in major agronomic crops. Our objective is to develop a tissue-culture independent protocol that enables manipulation of plastid genomes directly in plants yielding genetically stable seed progeny. We report that in planta excision of a plastid aurea bar gene (*bar<sup>au</sup>*) is detectable in greenhouse-grown plants by restoration of the green pigmentation in tobacco leaves. The P1 phage Cre or PhiC31 phage Int site-specific recombinase was delivered on the *Agrobacterium* T-DNA injected at the axillary bud site, resulting in the excision of the target-site flanked marker gene. Differentiation of new apical meristems was forced by decapitating the plants above the injection site. The new shoot apex differentiating at the injection site contained marker-free plastids in 30% to 40% of the injected plants, of which 7% transmitted the marker-free plastids to the seed progeny. The success of obtaining seed with marker-free plastids depended on repeatedly forcing shoot development from axillary buds, a process that was guided by the size and position of green sectors in the leaves. The success of in planta plastid marker excision proved that manipulation of the plastid genomes is feasible within an intact plant. Extension of the protocol to in planta plastid transformation depends on the development of new protocols for the delivery of transforming DNA encoding visual markers.

While the use of CRE/lox and phi3/att integrase/recombinases are exemplified herein, other recombinases and their cognate excision site sequences known by the skilled artisan and are encompassed within the scope of the present invention. See for example Table I in PCT/US04/06492.

The following materials and methods are provided to facilitate the practice of Example II.

Plant Materials and Growth Conditions

Nt-pSS33-1AA and Nt-pSS33-1BA tobacco plants used in this study carry a plastid-encoded spectinomycin resistance (*aadA*) gene and an aurea *bar<sup>au</sup>* plastid marker flanked with the 215-bp attP and 54-bp attB target sites (FIG. 7b). The attP and attB sites are recognized by the phiC31 phage Int recombinase. Nt-pSS42-11A and Nt-pSS42-18B are similar transplastomic plants, except that the *bar<sup>au</sup>* is flanked with 34-bp loxP sites recognized by the P1 phage Cre recombinase (FIG. 7c). These transplastomic *Nicotiana tabacum* cv. Petit Havana (tobacco) plants have been described (Tungsuchat-Huang et al. 2010).

The plants for injection were grown in 7.5 inches plastic pots with supplemental lighting (16 hours daylight) in the greenhouse until 10-12 inches tall in Pro-Mix general purpose growing medium Code 0432 (Premier Horticulture Inc., Quakertown, Pa.).

To identify seedlings with marker-free plastids, 200 to 300 seeds were germinated in 10"×10" square flat trays (Tungsuchat-Huang et al. 2010). The seedling phenotype was scored after 4 to 5 weeks as dark green or aurea.

#### *Agrobacterium* Injection

Buds were removed and then each node was injected at the bud site with *Agrobacterium tumefaciens* strain EHA101 carrying binary plasmids pKO31 or pKO117. Plasmid pKO31 carries in its T-DNA a plastid-targeted Cre recom-

binase and a plant-selectable gentamycin resistance (aacC1) gene (FIG. 7d) (Corneille et al. 2003). Plasmid pKO117 is a similar binary plasmid, other than it encodes a plastid targeted Int (FIG. 7e) (Lutz et al. 2004). *Agrobacterium* suspension cells were prepared as described (Lutz et al. 2006a, Lutz et al. 2006b). Briefly, *Agrobacterium* carrying the binary plasmids was inoculated in 100 ml YEB medium supplemented with 100 mg/L spectinomycin and 50 mg/L kanamycin and grown overnight at 27° C. 1 ml of the overnight culture was transferred into fresh YEB medium containing 10 mM MES, pH-adjusted to 5.6, 20 µM aceto-syringone, 100 mg/L spectinomycin and 50 mg/L kanamycin and grown overnight at 27° C. The bacterial cultures were sedimented by centrifugation and resuspended in MMA medium (supplemented with 200 µM acetosyringone) to a final OD<sub>600</sub>=2.4 and incubated at room temperature for at least 2-3 hours.

Prior to injection the Nt-pSS33 or Nt-pSS42 plants were heavily watered, then the shoots were decapitated and all buds were removed. Each node was then injected at the bud site at least three times using a 3-mL syringe with a 25G×1½ inches needle (Becton Dickinson, Franklin Lakes, N.J.). After the injection the plants were covered with plastic sheets to prevent desiccation and stored in low light at 25° C.-30° C. for 24 hours before moving them back into the greenhouse.

#### DNA Gel-Blot Analyses and Sequencing of PCR Products

Total cellular DNA was prepared from leaf tissue by the CTAB protocol (Murray et al. 1979). DNA gel-blot analysis was performed as described. Total cellular DNA was digested with the BamHI restriction enzyme and separated by electrophoresis in 0.8% agarose gel. The DNA was transferred to Hybond-N membranes (GE Healthcare, Piscataway, N.J.) and hybridized with a random-primed, P<sup>32</sup>-labeled rrn16 plastid targeting-region probe (ApaI-BamHI fragment) (Svab and Maliga 1993).

For sequencing the recombinant target sites, the intergenic region between rrn16 and 3'rps12/7 gene was amplified from total cellular DNA with primer F1/#816 (5'-GGCTTCAG-GCCGCCATCCACT-3', SEQ ID NO: 4) and R1/#T01 (5'-GTAGTTAATGGTAGGGTTACC-3', SEQ ID NO: 5) and sequenced with primer R1/#T01. The position of the F1 and R1 primers is shown in FIG. 7b,c.

#### Testing Leaf Sections for Gentamycin Resistance

Leaves from greenhouse plants were surface sterilized with 10x diluted Clorox bleach (0.6% sodium hypochlorite; 3 min) in a glass tray and then washed three times with sterile distilled water. The sterile leaves were blotted on a Whatman filter paper to absorb the excess water, then cut into 1 cm×1 cm squares and placed on RMOP media supplemented with 100 mg/L gentamycin (Carrer et al. 1990). The number of tested sections depended on sector size.

To test the feasibility of in planta plastid genome manipulation, we decided to excise the bar<sup>au</sup> gene with the P1 phage Cre or PhiC31 phage Int site-specific recombinase. Useful for this study were the transplastomic plants in which the bar<sup>au</sup> gene is flanked by recombinase target sites (Tungsuchat-Huang et al. 2010). The success of the project depended on obtaining shoots in which the marker-free plastids were at the leaf margins, indicating the presence of marker-free plastids in the second layer of the shoot apex, the source of germline cells in plants (Poethig 1989). To force regeneration of shoots from *Agrobacterium*-transformed cells, we decapitated the plants, removed the top two or three axillary buds, and injected the bud site with *Agrobacterium*.

## Results

### System for Detection of Recombinase-Mediated Plastid Marker Excision

We have designed a sensitive system to detect plastid marker excision in plants by the change of leaf color. The presence of the bar<sup>au</sup> gene confers a golden-yellow (aurea) leaf color due to interference with the expression of the plastid clpP gene (Kittiwongwattana et al. 2007). When the bar<sup>au</sup> marker gene is excised, green pigmentation is restored, providing a readily detectable visual phenotype for marker excision. In the Nt-pSS33 plastids the bar<sup>au</sup> gene is flanked by attB and attP sites (FIG. 7b) that are recognized by Int, the PhiC31 phage site-specific recombinase (Tungsuchat-Huang et al. 2010). In the Nt-pSS42 plastids the visual marker gene is flanked by loxP sites (FIG. 7c), which are the target sites of the P1 phage recombinase (Tungsuchat-Huang et al. 2010).

The recombinase genes were expressed from the T-DNA region of *Agrobacterium* binary transformation vectors: plasmid pKO31 (Corneille et al. 2003) and pKO117 (Lutz et al. 2004) encode a plastid-targeted Cre and Int, respectively (FIG. 7d, e). Plastid targeting was facilitated by fusing the recombinases with the rubisco small subunit transit peptide and five (pKO31) or 22 (pKO117) amino acids of the mature rubisco small subunit. Both vectors carry a gentamycin resistance (aacC1) gene. We used gentamycin resistance to distinguish recombinase delivery from a transiently expressed or stably integrated T-DNA by the absence or presence of the gentamycin resistance marker gene (Lutz et al. 2006a).

### Green Sectors Form on the Aurea Leaves After *Agrobacterium* Injection

De novo transformation of tissue culture cells with a plastid-targeted recombinase is the most common protocol to obtain marker-free transplastomic plants (Lutz and Maliga 2007). We report here a novel, second de novo transformation approach by directly transforming the germline cells in the shoot apex by injected *Agrobacterium* cells. During the injection the needles destroyed the shoot apex, thereby forcing the regeneration of new shoot meristems from the injected tissue (FIG. 8a). New shoots with dark-green sectors on the aurea leaf background appeared one to two weeks after the injection. The dark green sectors in some of the leaves were localized to the leaf margin of a single leaf (FIG. 8b), in others many dark-green excision sectors formed in the different developmental layers, but excision was still restricted to a single leaf (FIG. 8c,d). Localization of the sectors to a single leaf suggests that excision took place in the leaf primordia (Poethig 1989). In other plants dark-green sectors were present in multiple leaves indicating that the cells carrying bar<sup>au</sup> marker-free plastids were present in the shoot apex. The size of the dark-green sectors in some shoots was sizable, with half of the shoot apex made up of cells with dark-green plastids. The dark green cells in the variegated leaf in FIG. 8e derive from Layer 2 of the shoot apex (Poethig 1989). When the shoot was decapitated above the green leaf (arrow), the shoot developing from the axillary bud had a uniform dark-green color, with dark green plastids in both Layer 2 and 3 (FIG. 8f).

In two experiments a total of 147 plants were injected (Table 2). Out of 73 Cre-injected plants 32 (43%) developed shoots with green sectors, out of which 12 (16%) had dark green plastids in Layer 2 of the leaf (Table 2). Cells in Layer 2 of the leaf have the same genotype as the germline cells in the shoot apex indicating the potential for seed transmission (Poethig 1989). Injection of Nt-pSS33 plants with Int

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yielded plants with dark-green sectors at a similar frequency. Out of 74 Int-injected plants 20 plants (27%) developed shoots with green sectors, out of which 9 (12%) carried cells with marker-free plastids at the leaf margin indicating potential for seed transmission (Table 2).

TABLE 2

Frequency of in planta plastid marker excision in <i>Agrobacterium</i> -injected plants				
Experiment	Recombinase	No. of injected plants	No. of plants with sectors	No. of plants with green plastids in germline
I	Cre	50	19	3
	Int	50	18	8
II	Cre	23	13	9
	Int	24	2	1
I + II	Cre	73	32 (43%)	12 (16%)
	Int	74	20 (27%)	9 (12%)

#### DNA Analysis Confirms *bar<sup>au</sup>* Excision in the Green Sectors

Excision of *bar<sup>au</sup>* in the green sectors of variegated leaves was confirmed by DNA gel blot analyses using the plastid targeting region (*rrn16*) probe (FIG. 7a) (Kittiwongwattana et al. 2007). In the transplastomic aurea Nt-pSS42 plants the *rrn16* targeting region probe detects 3.8-kb and 1.6-kb fragments whereas Cre excision yields a 4.4-kb *bar<sup>au</sup>*-lacking fragment (FIG. 7c). A DNA gel blot analysis in the green sectors of injected Nt-pSS42 plants indicates a mixed *bar<sup>au</sup>*-containing and *bar<sup>au</sup>*-free ptDNA population (FIG. 9a). A similar analysis of the green sectors was carried out in the *Agrobacterium*-injected Nt-pSS3 plants. In the transplastomic aurea Nt-pSS3 plants the *rrn16* targeting region probe detects 3.8-kb and 1.8-kb fragments and Int excision yields a 4.5-kb ptDNA fragment lacking *bar<sup>au</sup>* (FIG. 7b). A DNA gel blot analysis in the nine dark-green sectors indicates mixed *bar<sup>au</sup>*-containing and *bar<sup>au</sup>*-free ptDNA population (FIG. 9a). The ratio of *bar<sup>au</sup>* containing and lacking fragments is variable. In two sectors (number 2 and 4) the *bar<sup>au</sup>* containing 3.8-kb fragment is almost absent.

Excision of *bar<sup>au</sup>* was confirmed by sequencing PCR-amplified excision products directly from the green sectors of the aurea leaves. The primers complementary to *aadA* and the ptDNA targeting region (F1 and R2 in FIG. 7b,c) selectively amplified the excision product. DNA sequence of the fragments from 14 Nt-pSS42 plants and 9 Nt-pSS33 green sectors revealed only the *aadA* gene promoter, one recombinant target site (*loxP* attL) and the plastid targeting region, as predicted.

#### Plastid Marker Excision is by Transiently Expressed or Stably Integrated Recombinase Genes

Excision of the plastid marker may be carried out by a transiently expressed or by a stably integrated recombinase. Transient or stable delivery can be distinguished by the absence or presence of the gentamycin resistance gene in the green sectors that are adjacent to the recombinase genes in the T-DNA (FIG. 7d,e). To determine the transient or stable mode of delivery, we tested the gentamycin resistance phenotype of the green sectors by culturing them on a medium containing gentamycin. Formation of green calli on the selective medium indicates gentamycin resistance and brown necrotic tissue gentamycin sensitivity (FIG. 10).

Testing 1 cm<sup>2</sup> dark green leaf sections revealed that eleven of the green sectors were gentamycin sensitive indicating marker excision by a transiently expressed recombinase, whereas explants from four sectors were gentamycin resistant indicating the presence of an integrated recombinase

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(T-DNA) copy (Table 3). Segregation for gentamycin resistance in eight of the 23 green sectors suggests marker excision by both a transiently expressed and stably integrated recombinase (FIG. 10c).

TABLE 3

Testing recombinase integration by expression of linked gentamycin resistance gene							
Nt-pSS42-11A/Cre				Nt-pSS33-BA/Int			
Leaf explants <sup>a</sup>	Gent-R <sup>b</sup>	Gent-S <sup>c</sup>	Total	Leaf explants <sup>a</sup>	Gent-R <sup>b</sup>	Gent-S <sup>c</sup>	Total
1-1.1	1	6	7	3-1	3	4	7
1-1.2	2	5	7	3-2	0	6	6
1-1.4	0	3	3	3-3	0	5	5
1-1.5	1	3	4	3-5	3	0	3
1-1.8	0	2	2	3-9	3	2	5
1-1.9	1	3	4	3-10	0	7	7
1-1.10	7	0	7	4-1	3	0	3
1-1.11	1	5	6	4-5	3	0	3
1-1.12	0	2	2	4-9	6	3	9
1-1.13	0	5	5				
1-4	0	2	2				
2-2	0	7	7				
2-7	0	2	2				
2-12	0	2	2				

<sup>a</sup>The first digit identifies a plant; the second a leaf and the third a green sector in the leaf. For example, Explant 1-1.1 was taken from Plant No. 1, leaf No. 1 and sector No. 1.

<sup>b</sup>Gentamycin resistant

<sup>c</sup>Gentamycin sensitive

#### Seed Transmission of Marker-Free Plastids

In dicots the second leaf layer is formed from the same cells that give rise to the germline. The phenotype of the second leaf layer can be judged by the color of the leaf mesophyll cells at the leaf margins (Poethig 1989). Based on the position of the green sectors in the leaf, inheritance of the marker-free plastids was expected in 21 (14%) of the 147 injected plants. Seed transmission of the *bar<sup>au</sup>* marker-free plastids was studied in detail in eleven of the lines: nine lines injected with the Cre (I1-2, I11-1, I11-5, I11-11, I2-2, I2-5, I2-9, I3-6) and two injected with Int (I3-2, I3-5). About 200 seeds from five capsules each was sown, and grown for four to five weeks, until the genotype of the seedlings could be judged. A typical result in FIG. 11 is shown with I3-2 seedlings (Nt-pSS33 excised with Int). Some of the capsules (number 1, 4) contained predominantly green, marker free plastids; others contained seed with pure *bar<sup>au</sup>* seedlings (number 5), or a mixed progeny (capsules 2, 3). DNA gel blot analyses confirmed absence of the *bar<sup>au</sup>* gene (FIG. 9b). Mixed *bar<sup>au</sup>*-free and *bar<sup>au</sup>*-containing ptDNA was detected only in the rare variegated seedlings (white arrow in FIG. 11). Out of the 11 lines tested, seed transmission of the marker-free plastids was shown in 6 lines (I11-1, I11-11, I2-9, I3-6, I3-2 and I3-5). DNA gel blot analyses indicated a homoplasmic population of marker-free plastids in the green seedlings in each of the six lines (FIG. 9b). Assuming seed transmission in 5 of the 10 non-tested clones, we expect seed transmission in 11 (7%) of the 147 injected plants.

#### Discussion

##### Plastid Marker Excision by Recombinases in Planta

Multiple protocols exist to obtain marker-free transplastomic plants including: repeat-mediated marker excision, transient cointegration, cotransformation-segregation and recombinase-mediated marker excision (Day and Goldschmidt-Clermont 2011, Lutz and Maliga 2007). Recombinase-mediated marker excision is simpler than the alterna-



tive protocols, because in this approach plastid transformation and marker excision are two separate processes. The transplastomes with target-site flanked marker genes are stable in the absence of recombinases, therefore the time of marker excision can be chosen at will (Tungsuchat-Huang et al. 2010). The marker excision protocol that we report here avoids tissue culture directly yielding marker-free seed, thus providing a facile alternative to tissue culture transformation (Corneille et al. 2001, Hajdukiewicz et al. 2001, Kittiwongwattana et al. 2007) or pollination (Corneille et al. 2001) as the means of introducing the recombinase gene. Due to transient recombinase expression, the nuclear genome remains unmodified in a significant number of marker-free events, thus eliminating the need to segregate away the recombinase gene in the seed progeny (see next section).

The process of in planta marker gene excision is relatively efficient. Plastid marker excision was obtained in 43% and 25% of the plants injected with the Cre and Int recombinases, respectively (Table 2). Out of these, about 14% of the injected shoots yielded sectors in leaf Layer 2, an indication that capsules from that branch will transmit the marker-free plastids to the seed progeny. Seed transmission of marker-free plastids was studied in eleven clones, representing plants regenerated from different injected plants: nine with the Cre and two with the Int recombinases. We identified marker-free plastids in six out of the eleven lines by testing about 200 seedlings in five capsules. The aurea *bar<sup>au</sup>* is a young leaf marker because the older leaves turn green. Thus, at the time of seed collection leaf color does not provide guidance as to which capsules to pick. Testing more capsules and individually marking capsule position relative to sectors should ensure recovery of seedlings with marker-free plastids in most clones.

DNA samples isolated from the green sectors of the injected plants were heteroplasmic, with mixed marker-free and marker-containing ptDNA (FIG. 9a). This may be due to a heteroplasmic state at the cellular level, or different ptDNA type being present in the three leaf layers. Interestingly, green marker-free seedlings in the seed progeny of these plants were homoplasmic, carrying only marker-free plastids (FIG. 9b). Apparently, plastid sorting in Layer 2 of the shoot apex, the source of germline cells, was complete by the time the flowers formed.

Some of the Clones with Marker-Free Plastids Carry no Integrated Recombinase Gene

The green sectors identified cells with marker-free plastids in the leaves, but provided no information about the integrase gene encoded in the T-DNA. Information about the relative frequency of plastid marker excision by transiently expressed and stably integrated recombinase was obtained by testing gentamycin resistance in the green leaf sectors. In many of the green sectors (11 out of 23), marker excision took place by a transiently expressed recombinase, as the tissue culture derived from the green sectors was gentamycin sensitive, lacking the nuclear marker carried on the T-DNA (Table 3). Four green sectors yielded gentamycin resistant cultures indicating the presence of an integrated recombinase gene on an T-DNA. Eight green sectors segregated for gentamycin resistance indicating *bar<sup>au</sup>* excision by the expression of both transiently expressed and stably integrated recombinase genes (Table 3). Based on these data we expect that about half of lines lack an integrated recombinase in the nucleus.

We reported plastid marker excision by a transiently expressed Cre recombinase earlier using a more labor intensive protocol, random screening of plants regenerated from

an Agroinfiltrated tobacco leaf (Lutz et al. 2006a). The 10% frequency of plants with marker-free plastids, and no integrated recombinase gene in the nucleus, was surprisingly high. Using a visual plastid marker in Agroinjected plants, or as a screening tool in a randomly regenerated sample, should simplify this task.

### Example III

#### In Planta Transformation of Plastid Genomes

Our success with in planta marker excision lead us to consider what steps would enable in planta plastid transformation. There are two fundamental elements to transformation: DNA delivery and the identification of transgenic events. We developed two aurea plastid markers that are suitable for visual identification: the *bar<sup>au</sup>* (Kittiwongwattana et al. 2007) and the *aadA<sup>au</sup>* genes (Tungsuchat-Huang et al. 2011). Because the *bar<sup>au</sup>* gene is not suitable for the recovery of transplastomic clones in tissue culture (Lutz et al. 2001, Ye et al. 2003), the *bar<sup>au</sup>* gene currently is a component of a two-gene visual marker system, in which *bar<sup>au</sup>* is introduced into plastids by selection for a linked *aadA* gene. In applications for in planta plastid transformation *bar<sup>au</sup>* or *aadA<sup>au</sup>* alone is sufficient as a visual marker. Thus, the plastid transformation vector intended for in planta applications will have *bar<sup>au</sup>* or *aadA<sup>au</sup>* as a visual marker and one or more gene of interest, flanked by ptDNA fragments to target the insertion of the transgenes into the plastid genome. The transformation vector will be delivered to cells that are suitably positioned in the plant body to form a shoot apex. When the shoot apex derives from DNA-treated cells sprouts, the transplastomic clones will be recognized as aurea leaf sectors. Collection of transplastomic seed will be guided by the position of aurea sectors in the plants, as described above.

As for the method of DNA delivery, the biolistic protocol could be directly applied to an axillary bud site (Finer et al. 1992, Sanford et al. 1993, Ye et al. 1990). This could be accomplished with hand-held versions of the biolistic gun, such as the Helios Gene Gun. In one approach, the bud could be excised, and cells in the proper anatomical position bombarded in a PDS-1000He biolistic device. The bud so treated is then grafted onto a proper rootstock to grow shoots from the bombarded tissue. Alternatively, to provide a suitable vacuum, the current version of the biolistic gun could be modified to partially enclose the plant's body in a vacuum chamber. An alternative method for DNA delivery would be re-engineering the *Agrobacterium*'s molecular machinery to target the T-DNA to plastids. If successful, Agroinjection could be applied for in plantaplastid transformation as it is done today for in planta excision of plastid marker genes.

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<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
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<400> SEQUENCE: 7

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Val Val Gly Val Ile Glu Arg His Leu Glu Pro Thr Leu Leu Ala Val
 35            40            45
His Leu Tyr Gly Ser Ala Val Asp Gly Gly Leu Lys Pro His Ser Asp
 50            55            60
Ile Asp Leu Leu Val Thr Val Thr Val Arg Leu Asp Glu Thr Thr Arg
 65            70            75            80
Arg Ala Leu Ile Asn Asp Leu Leu Glu Thr Ser Ala Ser Pro Gly Glu
 85            90            95
Ser Glu Ile Leu Arg Ala Val Glu Val Thr Ile Val Val His Asp Asp
100           105           110
Ile Ile Pro Trp Arg Tyr Pro Ala Lys Arg Glu Leu Gln Phe Gly Glu
115           120           125
Trp Gln Arg Asn Asp Ile Leu Ala Gly Ile Phe Glu Pro Ala Thr Ile
130           135           140
Asp Ile Asp Leu Ala Ile Leu Leu Thr Lys Ala Arg Glu His Ser Val
145           150           155           160
Ala Leu Val Gly Pro Ala Ala Glu Glu Leu Phe Asp Pro Val Pro Glu
165           170           175
Gln Asp Leu Phe Glu Ala Leu Asn Glu Thr Leu Thr Leu Trp Asn Ser
180           185           190
Pro Pro Asp Trp Ala Gly Asp Glu Arg Asn Val Val Leu Thr Leu Ser
195           200           205
Arg Ile Trp Tyr Ser Ala Val Thr Gly Lys Ile Ala Pro Lys Asp Val
210           215           220
Ala Ala Asp Trp Ala Met Glu Arg Leu Pro Ala Gln Tyr Gln Pro Val
225           230           235           240
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Ala Ser Arg Ala Asp Gln Leu Glu Glu Phe Val His Tyr Val Lys Gly
260           265           270

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Glu Ile Thr Lys Val Val Gly Lys Glu Gln Lys Leu Ile Ser Glu Glu  
 275 280 285

Asp Leu  
 290

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What is claimed is:

1. An isolated nucleic acid encoding a visibly detectable selectable marker for identification of transformed plastids, wherein said nucleic acid encodes aadA<sup>au</sup> protein of SEQ ID NO: 7 and confers the aurea phenotype under culture and greenhouse conditions.

2. A plant transformation vector comprising the isolated nucleic acid of claim 1 suitable to transform plastids of higher plants.

3. The vector of claim 2, comprising a multiple-cloning site suitable for insertion of a heterologous nucleic acid encoding a protein of interest.

4. The vector of claim 3, which is pKMS8 (SEQ ID NO: 6).

5. The vector of claim 3, comprising a heterologous nucleic acid encoding a molecule selected from the group consisting of a drought resistance protein, an herbicide resistance protein, a cytokine, an siRNA, a miRNA, shRNA, an antisense RNA, an antibody, a hormone, a receptor and a ligand.

6. A plant cell transformed with the vector of claim 3.

7. A plant comprising the cell of claim 6.

8. A method for obtaining transplastomic plants, said method comprising introducing the nucleic acid of claim 1 into a plant cell, selecting those transformed cells which exhibit a golden leaf phenotype and spectinomycin resistance under culture and green house conditions, regenerating shoots from said transformed cells, and rooting said shoots in soil under conditions suitable to generate said transplastomic plant.

9. A method for obtaining transplastomic plants, said method comprising introducing the vector of claim 3 into a plant cell, selecting those transformed cells which exhibit a golden leaf phenotype and spectinomycin resistance under culture and greenhouse conditions, regenerating shoots from said transformed cells, and rooting said shoots in soil under conditions suitable to generate said transplastomic plant.

10. A transplastomic plant obtained by the method of claim 9.

11. The plant of claim 10 which is a monocot or a dicot.

\* \* \* \* \*